



King's Research Portal

DOI:

[10.1016/j.immuni.2015.02.003](https://doi.org/10.1016/j.immuni.2015.02.003)

Document Version

Publisher's PDF, also known as Version of record

[Link to publication record in King's Research Portal](#)

Citation for published version (APA):

Brown, C. C., Esterhazy, D., Sarde, A., London, M., Pullabhatla, V., Osma-Garcia, I., Al-Bader, R., Ortiz, C., Elgueta, R., Arno, M., de Rinaldis, E., Mucida, D., Lord, G. M., & Noelle, R. J. (2015). Retinoic Acid is essential for Th1 cell lineage stability and prevents transition to a th17 cell program. *Immunity*, 42(3), 499-511. <https://doi.org/10.1016/j.immuni.2015.02.003>

Citing this paper

Please note that where the full-text provided on King's Research Portal is the Author Accepted Manuscript or Post-Print version this may differ from the final Published version. If citing, it is advised that you check and use the publisher's definitive version for pagination, volume/issue, and date of publication details. And where the final published version is provided on the Research Portal, if citing you are again advised to check the publisher's website for any subsequent corrections.

General rights

Copyright and moral rights for the publications made accessible in the Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognize and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the Research Portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the Research Portal

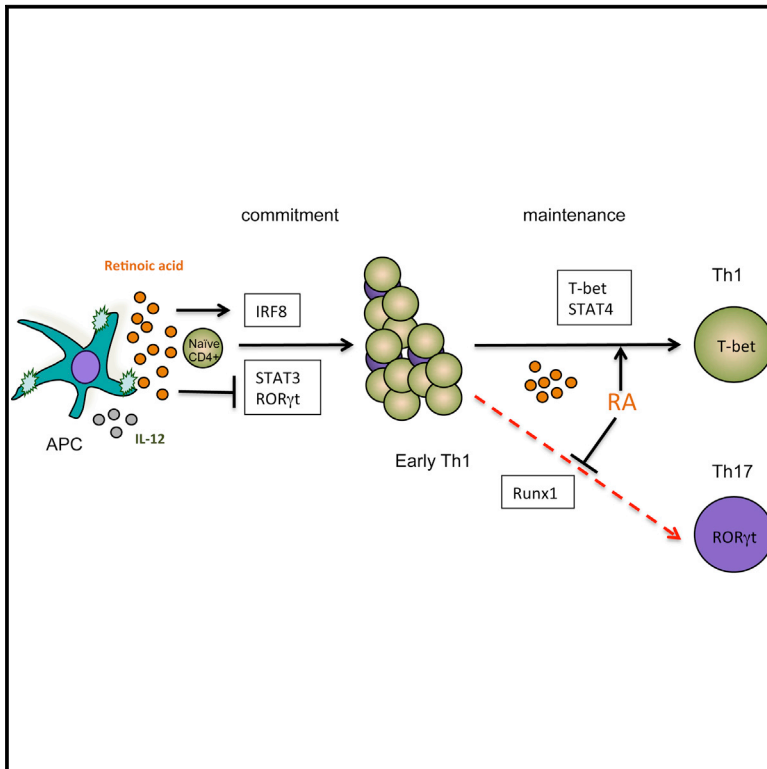
Take down policy

If you believe that this document breaches copyright please contact librarypure@kcl.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

Immunity

Retinoic Acid Is Essential for Th1 Cell Lineage Stability and Prevents Transition to a Th17 Cell Program

Graphical Abstract



Authors

Chrysothemis C. Brown,
Daria Esterhazy, ..., Graham M. Lord,
Randolph J. Noelle

Correspondence

chrysothemis.brown@kcl.ac.uk (C.C.B.),
rjn@dartmouth.edu (R.J.N.)

In Brief

Maintenance of T helper (Th)-cell identity is critical for appropriate immune responses; however, the factors that regulate Th-cell plasticity are unresolved. Brown et al. show that retinoic-acid signaling confers Th1 cell stability and restrains their conversion to Th17 cells.

Highlights

- Retinoic acid (RA) stabilizes Th1 fate commitment
- Signaling through RA receptor α (RAR α) activates enhancers of Th1-cell-lineage-specifying genes
- RA-RAR α represses Th17-cell genes in Th1 cells and constrains Th1-cell plasticity
- RA-RAR α prevents development of pathogenic Th17 cells in vivo



Brown et al., 2015, Immunity 42, 499–511
March 17, 2015 ©2015 The Authors
<http://dx.doi.org/10.1016/j.immuni.2015.02.003>

CellPress

Retinoic Acid Is Essential for Th1 Cell Lineage Stability and Prevents Transition to a Th17 Cell Program

Chrysothemis C. Brown,^{1,*} Daria Esterhazy,² Aurelien Sarde,¹ Mariya London,² Venu Pullabhatla,³ Ines Osma-Garcia,¹ Raya al-Bader,¹ Carla Ortiz,¹ Raul Elgueta,¹ Matthew Arno,⁴ Emanuele de Rinaldis,^{3,5} Daniel Mucida,² Graham M. Lord,^{1,3} and Randolph J. Noelle^{1,6,*}

¹Division of Transplantation Immunology and Mucosal Biology, King's College London, London SE1 9RT, UK

²Laboratory of Mucosal Immunology, The Rockefeller University, New York, NY 10065, USA

³National Institute for Health Research Biomedical Research Centre at Guy's & St Thomas' National Health Service Foundation Trust and King's College London, London SE1 9RT, UK

⁴Genomics Centre, King's College London, London SE1 9NH, UK

⁵Division of Cancer Studies, School of Medicine, King's College London, Guy's Hospital, London SE1 9RT, UK

⁶Department of Microbiology and Immunology, Dartmouth Medical School, Lebanon, NH 03756, USA

*Correspondence: chrysothemis.brown@kcl.ac.uk (C.C.B.), rjn@dartmouth.edu (R.J.N.)

<http://dx.doi.org/10.1016/j.immuni.2015.02.003>

This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

SUMMARY

CD4⁺ T cells differentiate into phenotypically distinct T helper cells upon antigenic stimulation. Regulation of plasticity between these CD4⁺ T-cell lineages is critical for immune homeostasis and prevention of autoimmune disease. However, the factors that regulate lineage stability are largely unknown. Here we investigate a role for retinoic acid (RA) in the regulation of lineage stability using T helper 1 (Th1) cells, traditionally considered the most phenotypically stable Th subset. We found that RA, through its receptor RAR α , sustains stable expression of Th1 lineage specifying genes, as well as repressing genes that instruct Th17-cell fate. RA signaling is essential for limiting Th1-cell conversion into Th17 effectors and for preventing pathogenic Th17 responses *in vivo*. Our study identifies RA-RAR α as a key component of the regulatory network governing maintenance and plasticity of Th1-cell fate and defines an additional pathway for the development of Th17 cells.

INTRODUCTION

Functional plasticity within cells of the innate and adaptive immune system increases the breadth of response to pathogens while also limiting responses detrimental to the host. CD4⁺ T cells diversify into distinct effector subsets upon antigenic stimulation. Cytokines and other microenvironmental factors present during T-cell priming direct differentiation via induction of lineage specifying transcription factors (TFs): T-bet is the “master” regulator for T helper 1 (Th1) cells, ROR γ t for Th17 cells, and GATA3 directs the Th2 program. *In vivo*, the presence of cells that express TFs and cytokines from opposing Th line-

ages indicates flexibility between those subsets. Late-stage developmental plasticity is potentially perilous: interferon- γ (IFN- γ) Th17 cells have been implicated in several human autoimmune diseases including inflammatory bowel disease (Annunziato et al., 2007), juvenile idiopathic arthritis (Nistala et al., 2010), and multiple sclerosis (Kebir et al., 2009); ex-Foxp3⁺ Th17 cells play a pathogenic role in rheumatoid arthritis (Komatsu et al., 2014); and interleukin-17 (IL-17⁺) Th2 cells have been positively linked to the severity of asthma (Irvin et al., 2014). Elucidating the developmental pathways for these hybrid cells and identifying the factors that regulate Th-cell stability are therefore of critical importance.

Initial lineage specification is driven by cytokines, which activate signal transducer and activator of transcription (STAT) proteins: expression of T-bet is driven by IFN- γ -STAT1 and IL-12-STAT4 (Schulz et al., 2009); ROR γ t by STAT3 downstream of IL-6, IL-21, and IL-23 (Zhou et al., 2007). Less is known about the molecular mechanisms that sustain lineage identity. Epigenetic modifications stabilize gene expression and as such, are thought to play a key role in the maintenance of cell-fate commitment. However, the factors that co-ordinate chromatin changes with evolving TF networks in differentiating Th cells are not fully defined. One candidate is the vitamin A metabolite, retinoic acid (RA). RA is known to play a key role in directing the lineage fate of hematopoietic stem cells (Chanda et al., 2013), dendritic cells (DCs) (Klebanoff et al., 2013), innate lymphoid cells (ILCs) (Spencer et al., 2014), and CD4⁺ T cells (Reis et al., 2013) through activation of the nuclear RA receptor (RAR). In addition to its classical role as a transcriptional regulator, recent studies in embryonic stem cells have identified RA-RAR as an epigenetic regulator (Kashyap et al., 2013; Urvalek and Gudas, 2014). RA synthesis is dynamically controlled at sites of T-cell priming during inflammation, where RA signaling on T cells has been demonstrated (Aoyama et al., 2013; Pino-Lagos et al., 2011). These studies suggest a potential role for RA in Th-cell plasticity. Indeed, RA is critical for Th1-cell immunity (Hall et al., 2011; Pino-Lagos et al., 2011) and RA has also been implicated in Th17-cell differentiation where its impact appears to be

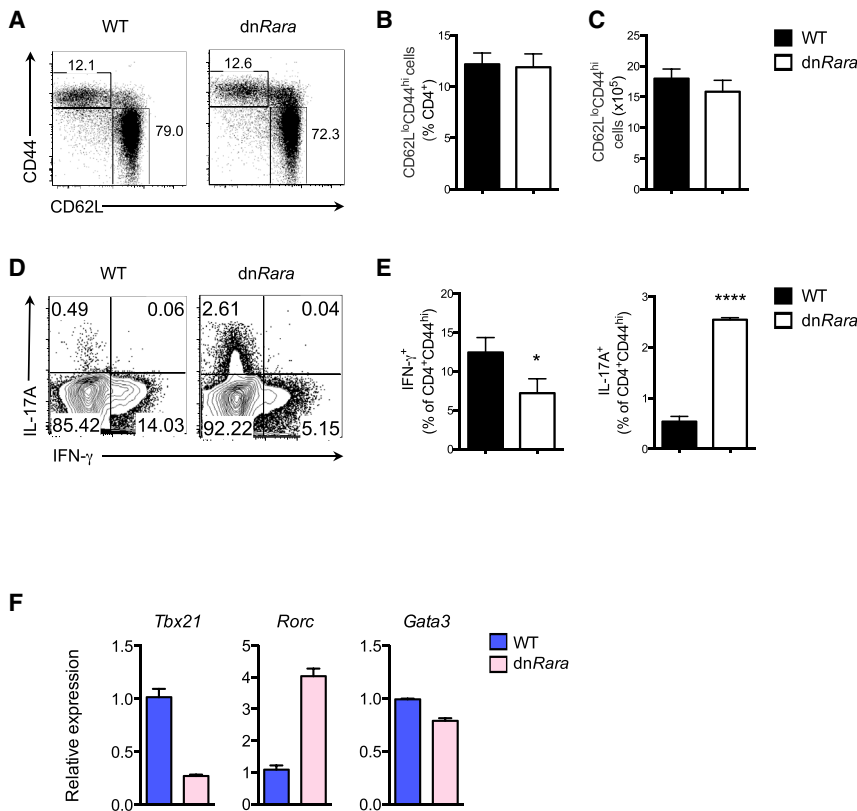


Figure 1. RA Controls the Balance between Th1 and Th17 Effector Cells

(A) Splenic CD4⁺ T cells from dnRara and WT littermate control mice. Numbers indicate percentage CD62L^{lo}CD44^{hi} cells (top left) or CD62L^{hi}CD44^{lo} T cells (bottom right) gated on CD4⁺ cells. (B) Frequency and total number (C) of CD62L^{lo}CD44^{hi} in the CD4⁺ T-cell population in WT and dnRara mice (n = 3 or 4 per group). (D) Intracellular IFN-γ and IL-17A expression in splenic CD4⁺CD44^{hi} T cells after stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin. (E) Statistical data from cells as in (D). (F) Quantitative real-time PCR analysis of *Tbx21*, *Rorc*, and *Gata3* in splenic CD4⁺CD62L^{lo}CD44^{hi} cells (as in 1A), sorted by flow cytometry. Data are from two or three independent experiments with similar results. Mean ± SEM, *p < 0.05; ****p < 0.0001. See also Figure S1.

dose dependent: physiological concentrations of RA enhance Th17-cell differentiation in vitro (Takahashi et al., 2012), yet administration of higher concentrations of RA both in vitro and in vivo negatively regulates Th17-cell responses (Mucida et al., 2007; Takahashi et al., 2012; Xiao et al., 2008). Although RARα has been identified as the critical mediator of RA actions in CD4⁺ T cells (Hall et al., 2011), to date a comprehensive analysis of the transcriptional targets of RARα in CD4⁺ T cells has not been reported and the mechanism by which RA regulates these distinct Th-cell fates remains unresolved.

Here we show that RA-RARα is critical for maintenance of the Th1-cell lineage. Loss of RA signaling in Th1 cells resulted in the emergence of hybrid Th1-Th17 and Th17 effector cells. Global analysis of RARα binding and enhancer mapping revealed that RA-RARα directly regulated enhancer activity at Th1-cell-lineage-defining genes while repressing genes that regulate Th17-cell fate. In the absence of RA signaling, infectious and oral antigen induced inflammation resulted in impaired Th1-cell responses with deviation toward a Th17-cell phenotype. These findings identify RA-RARα as a key regulatory node that acts to sustain the Th1-cell response while repressing Th17-cell fate.

RESULTS

RA-RARα Regulates the Balance between Th1 and Th17 Cells

To directly assess the role of RA in Th-cell differentiation in vivo we used mice carrying a sequence encoding a dominant-

negative form of the RA receptor RARα (RARα403) targeted to ROSA26 downstream of a loxP-flanked “stop” (Isl) cassette. As shown previously (Pino-Lagos et al., 2011), interbreeding with mice expressing Cre recombinase from the *Cd4* promoter generates *Cd4^{cre}dnRara^{Isl/Isl}* progeny (dnRara mice)

in which RA signaling is abrogated within the T-cell compartment. In contrast to *Rara*^{−/−} mice, expression of this dnRARα disrupts the RA dependent activity of RARα while retaining the ligand independent effects, allowing the specific analysis of RA-dependent functions.

To investigate the role of RA in the generation of Th-cell subsets under steady-state conditions, we determined the expression of cytokines within CD4⁺ T cells with an activated CD44^{hi} phenotype. Examination of the peripheral CD4⁺ T-cell compartment revealed equivalent frequencies and absolute numbers of CD44^{hi}CD62L^{lo}CD4⁺ memory cells in 8-week-old dnRara mice and in Cre[−], wild-type (WT), littermate controls (Figures 1A–1C). dnRara effector cells displayed reduced production of IFN-γ compared to their WT counterparts with a >5-fold increase in the frequency of IL-17⁺ cells (Figures 1D and 1E). Examination of transcripts for the signature lineage-determining TFs showed reduced mRNA expression of *Tbx21* and significantly higher expression of *Rorc* in dnRara effector CD4⁺ T cells (Figure 1F). Loss of RA signaling had no impact on Th2 effectors with equivalent levels of *Gata3* expression between dnRara and WT mice (Figure 1F) and similar frequencies of IL-4 producing CD4⁺ T cells (data not shown).

The frequency and numbers of Foxp3⁺ T cells in the periphery and thymus of dnRara mice were similar to control mice (Figures S1A and S1B), indicating that the increase in Th17 cells was not a consequence of reciprocal regulation by RA of Foxp3⁺CD4⁺ T cells and Th17 cells (Mucida et al., 2007). Therefore it is likely that under steady-state conditions RA is critical for differentiation of Th1 cells, while also limiting the differentiation of Th17 cells.

RA Promotes Th1-Cell Differentiation and Inhibits Development of Th17 Cells from Th1 Cell Precursors

We considered two alternative explanations of why *dnRara* mice exhibit reduced memory effector Th1 cells, in parallel with enhanced Th17 cells. The first possibility was that RA is required for the development of Th1 cells while independently suppressing the primary differentiation of Th17 cells. The alternative possibility was that RA is critical in restraining conversion of Th1 cells to Th17 cells. In order to resolve these two possibilities, naive CD4⁺ T cells were differentiated in the presence of Th1 or Th17 polarizing cytokines. *dnRara* expressing CD4⁺ T cells differentiated under Th1 cell conditions showed a markedly reduced capacity for IFN- γ production (Figure 2A). Diminished cytokine production was not a consequence of impaired proliferative responses as naive CD4⁺ T cells differentiated under Th1-cell conditions showed robust proliferation, equivalent to WT cells (Figure S2A). In addition, upregulation of the activation markers CD25 and CD44 indicated that *dnRara* T cells were not impaired in their ability to differentiate into effector cells (Figure S2B). Analysis of TF expression showed that ablating RA signaling resulted in a dramatic reduction in the expression of T-bet in CD4⁺ T cells differentiated under Th1-cell conditions (Figure 2B). Strikingly, a substantial proportion of *dnRara* Th1 cells expressed ROR γ t and co-expression of T-bet and ROR γ t was observed at the single-cell level. Although we did not observe intracellular IL-17A in cells following brief stimulation with phorbol myristate (PMA) and ionomycin, analysis of supernatants from Th1 polarized cells, reactivated on day 6 of culture on anti-CD3 and anti-CD28 coated plates for 24 hr in non-polarizing media, showed increased expression of IL-17A alongside other Th17-cell-associated cytokines (IL-21 and IL-22) (Figure 2C). Furthermore, mRNA analysis of *dnRara* Th1 polarized cells revealed dramatic increases in expression of key signature Th17-cell genes (Figure 2D). Notably, these Th1 cells displayed the hallmarks of pathogenic Th17 cells with high amounts of *Il23r* expression but reduced amounts of IL-10 mRNA and protein (Figures 2C and 2D) (Basu et al., 2013).

In order to assess whether enhanced Th17 responses were a general feature of CD4⁺ T cells in which RA signaling is disrupted, naive CD4⁺ T cells from *dnRara* mice were differentiated under Th17 polarizing conditions. In contrast to our observations above, we did not observe an increase in the frequency of IL-17⁺ cells in *dnRara* mice during primary differentiation into Th17 cells (Figure S2C), suggesting that RA restrains Th17-cell differentiation only in the context of a Th1 polarizing cytokine milieu. In support of this, ROR γ t expression was not observed in *dnRara* expressing naive CD4⁺ T cells differentiated under Th0 or Th2 conditions (Figure S2D).

The simultaneous expression of ROR γ t and T-bet in *dnRara* Th1 cells suggested that RA-RAR α might act to constrain the deviation of Th1 committed cells toward the Th17-cell lineage. To determine whether the ROR γ t⁺ cells represented a distinct T-cell population that arose directly from naive CD4⁺ T cells or from previously committed Th1 cells, we interbred *lfrng*^{eYFP} (Great) reporter mice with the *dnRara* mice to allow the tracking of IFN- γ ⁺ cells. Naive CD4⁺ T cells from *dnRara-lfrng*^{eYFP} or littermate control mice were activated under Th1 polarizing conditions. On day 7 of culture, eYFP⁺ (IFN- γ ⁺) cells were FACS sorted and underwent genome-wide expression analysis. Key signature

Th17-cell genes, including Th17-cell cytokines and receptors for cytokines that promote Th17-cell differentiation (*Il17f*, *Il21*, *Il1r1*, *Il6ra*, and *Il23r*), were highly expressed in *dnRara* IFN- γ expressing cells relative to WT mice, confirming a hybrid Th1-Th17-cell phenotype (Figure 2E). Of note, these Th1-Th17 cells retained high expression of *Il12rb2* and *Cxcr3* mRNA, equivalent to WT Th1 cells, while also expressing *Il23r* (Figure S2E). Genes associated with the Th2-cell subset such as *Gata3* and *Il4* were also dysregulated in *dnRara* Th1 cells consistent with a role for T-bet in repression of GATA3 (Zhu et al., 2012). These findings show that, in the absence of RA signaling, committed Th1-cell precursors can give rise to cells with a Th17-cell expression signature providing a new perspective on the origins of Th1-Th17 cells. Collectively, these data demonstrate that RA is not only required for Th1-cell differentiation but is also critical in suppressing Th17-cell development in Th1 polarized cells.

RA-RAR α Is Required for Late-Phase, STAT4-Dependent T-bet Expression in Th1 Cells

Early expression of T-bet following TCR activation is dependent on IFN- γ , whereas late expression of T-bet (post-termination of TCR signaling) has been shown to be dependent on IL-12 (Schulz et al., 2009). To distinguish a requirement for RA signaling in Th1-cell commitment from maintenance of Th1-cell fate, we examined the kinetics of T-bet expression in naive CD4⁺ T cells cultured under Th1 polarizing conditions. Induction of T-bet was observed with comparable amounts of T-bet expression between WT and *dnRara* T cells at day 3 of culture, indicating that RA-RAR α signaling is not required for early Th1 lineage commitment (Figure 3A). However, T-bet expression was not sustained in *dnRara* Th1 cells, with substantially diminished expression of T-bet by day 5 of culture. Given that IFN- γ promotes T-bet expression, the expression of T-bet was examined in the presence of recombinant IFN- γ , in order to avoid potential indirect effects caused by reduced IFN- γ production in *dnRara* Th1 cells. Exogenous IFN- γ enhanced early T-bet expression in both *dnRara* and WT Th1 cells but did not rescue the late (>72 hr) impairment in T-bet expression (Figure 3A). IFN- γ signaling, as measured by STAT1 phosphorylation, was not impaired at either time point (data not shown).

The late IL-12-dependent peak of T-bet expression observed in the presence of blocking IFN- γ antibodies was abrogated in *dnRara* Th1-cell polarized cells (Figure 3A) suggesting impaired STAT4 activity. At day 3 of culture, comparable amounts of phosphorylated STAT4 (pSTAT4) were observed between *dnRara* and WT mice. By contrast, at day 6 of culture, IL-12 induced pSTAT4 was markedly impaired in *dnRara* T cells (Figure 3B) despite comparable expression of IL-12R β 2 mRNA and protein expression and increased expression of *Il12rb1* mRNA (Figure 3C and 3D). Analysis of *Stat4* expression, demonstrated impaired induction of *Stat4* in the absence of RA signaling (Figure 3E) with reduced amounts of total STAT4 protein (Figure 3F). These findings suggest that the observed reduction in pSTAT4 in *dnRara* Th1 cells is a consequence of diminished STAT4 expression. Consistent with deviation toward the Th17-cell lineage, we observed enhanced pSTAT3 activity in Th1-cell polarized *dnRara* cells with an increased ratio of pSTAT3/pSTAT4 (Figures S3A and S3B).

To evaluate whether the impairment in T-bet and STAT4 expression correlated with changes in IFN- γ , the time course

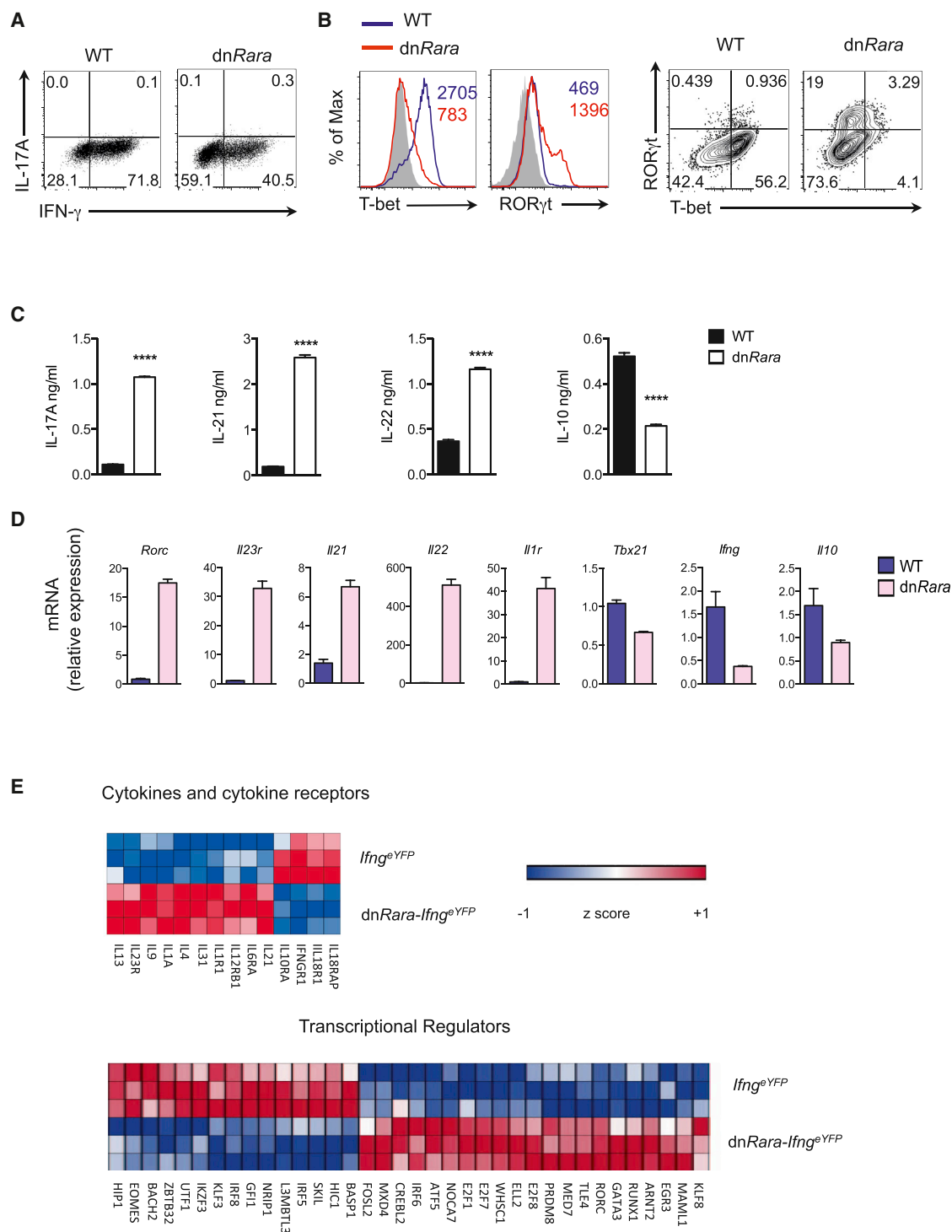


Figure 2. RA Signaling Required for Th1-Cell Differentiation and Repression of Th17-cell Fate in Th1-Cell Precursors

Sorted naive CD4⁺ T cells from *dnRara* or WT mice were cultured under Th1 conditions for 6 days.

(A) Intracellular expression of IFN- γ and IL-17A following stimulation with PMA and ionomycin.

(B) T-bet and ROR γ t expression. Grey histograms indicate staining for *Tbx21*^{-/-} (left panel) or isotype control antibody (right panel). Numbers show MFI. Numbers in quadrants represent percent cells in each.

(C) Amount of IL-17A, IL-21, IL-22, and IL-10 in supernatants following restimulation of cells as in (A) with α -CD3 and α -CD28 for 24 hr as measured by multiplex bead array. Triplicate culture wells.

(D) Quantitative real time PCR analysis of Th1 and Th17-cell signature cytokine and TF genes following stimulation with PMA and ionomycin.

(legend continued on next page)

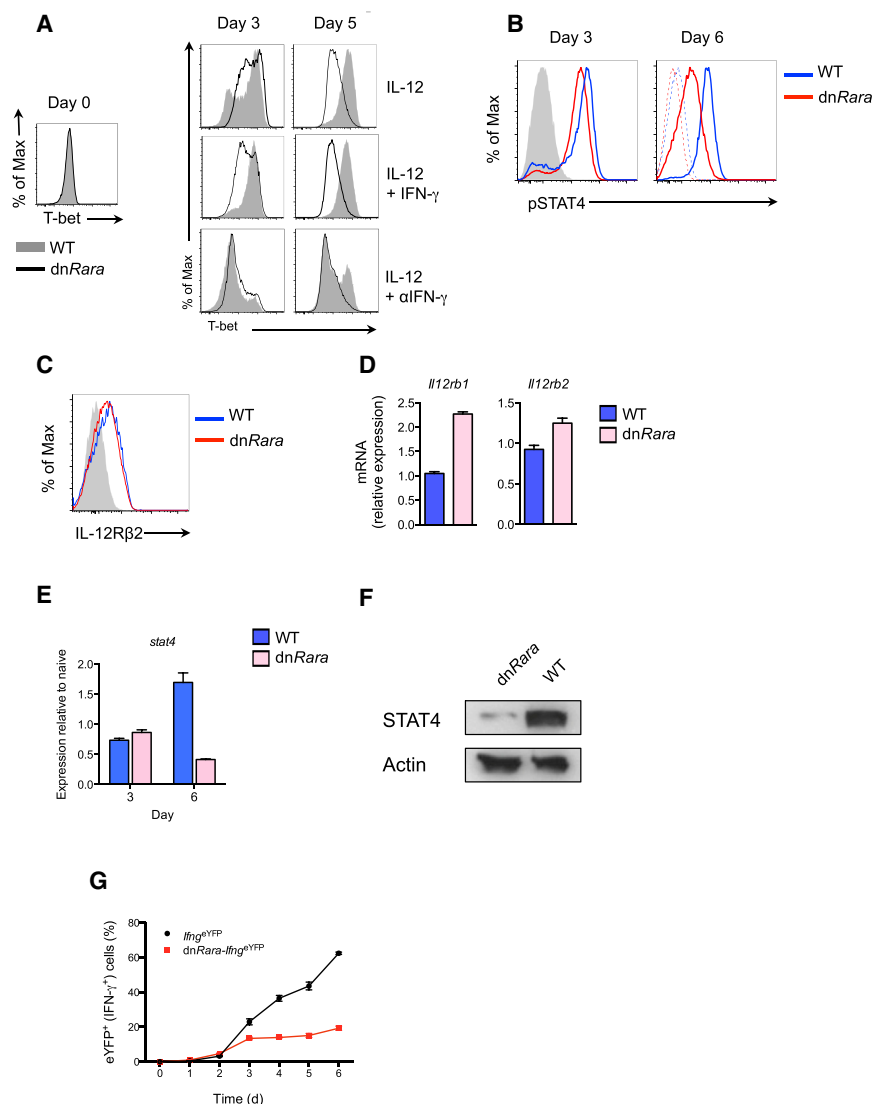


Figure 3. RA Required for Late Phase T-bet Expression

(A) Naive CD4 $^{+}$ T cells from dnRara and WT mice were differentiated under Th1 conditions with combinations of IFN- γ or IFN- γ antibody. T-bet expression analyzed at the indicated time points. Histograms gated on CD4 $^{+}$ T cells.

(B) Flow cytometric analysis of STAT4 phosphorylation in naive CD4 $^{+}$ T cells from dnRara and WT mice differentiated under Th1 conditions. Cells analyzed directly from culture after 3 days (left panel) or on day 6 following treatment with (solid lines) or without (dashed lines) 25 ng/ml IL-12 for 30 min (right panel). Shaded histogram displays pSTAT4 staining in cells cultured under Th0 conditions.

(C) Cell-surface expression of IL-12R β 2 on day 6 of culture.

(D) Quantitative real-time PCR analysis of *Il12rb1* and *Il12rb2* on day 6.

(E) Quantitative real-time PCR analysis of *Stat4* in Th1 polarized cells at indicated time points. Expression relative to naive CD4 $^{+}$ T cells.

(F) Western blot analysis of total STAT4 protein on day 6 of Th1 culture.

(G) Naive CD4 $^{+}$ T cells from dnRara-*lflng* eYFP and control mice were activated under Th1 conditions. Frequency of IFN- γ^{+} (eYFP $^{+}$) cells at indicated time points, gated on viable CD4 $^{+}$.

Data representative of two to three independent experiments. Mean \pm SEM.

See also Figure S3.

of IFN- γ expression following initiation of Th1-cell polarization was analyzed in naive dnRara-*lflng* eYFP expressing CD4 $^{+}$ T cells. The kinetics of IFN- γ induction, as measured by frequency of eYFP $^{+}$ cells, closely mirrored WT cells during the first 72 hr of culture but expression was not sustained in the absence of RA signaling (Figure 3G). Collectively, these data show that RA plays a temporal role in Th1 differentiation, maintaining Th1-cell commitment through regulation of T-bet and STAT4.

RA-RAR α Regulates Th1-Cell Plasticity

Alterations in the stable expression of lineage-determining TFs are thought to underlie Th-cell stability or plasticity. The emergence of Th1-Th17 cells together with the loss of T-bet expression, sug-

gested a role for RA in the regulation of Th1-cell plasticity. However, diminished T-bet and STAT4 activity from day 3 of primary Th1-cell differentiation prevented assessment of lineage stability in fully differentiated Th1 cells. To determine whether RA-RAR α was required for long-term Th1-cell fate, we differentiated naive CD4 $^{+}$ T cells from dnRara $^{ls/ls}$ mice under Th1-cell conditions, treated them with TAT-Cre (Wadia et al., 2004) on days 5 and 7, and restimulated them under Th1-cell conditions for a further 5 days. The temporal loss of RA signaling in Th1 cells resulted in decreased T-bet expression with a reciprocal increase in ROR γ t expression (Figure 4A). \sim 50% of cells expressed ROR γ t, which suggests that ongoing RA-RAR α activity is critical for sustaining T-bet and suppressing Th17-cell fate. Alterations in the lineage determining TFs did not impact on the cytokine phenotype (Figure S4A). This might in part reflect T-bet independent regulation of the *lflng* locus at late stages in Th1-cell development.

To further examine the role of RA in Th1-cell stability, naive CD4 $^{+}$ T cells from *lflng* eYFP mice were differentiated under

(E) Naive CD4 $^{+}$ T cells from dnRara-*lflng* eYFP and *lflng* eYFP mice were cultured under Th1 conditions. IFN- γ (eYFP $^{+}$) cells were sorted on day 7 following stimulation with PMA and ionomycin. Heatmaps displaying the fold changes of genes that were differentially expressed (fold change > 1.5, $p < 0.05$) for selected cytokines or cytokine receptors (upper panel) and TFs (lower panel). Samples from three independent experiments.

Representative data of at least three (A and B) or two (C and D) independent experiments. Mean \pm SEM.

See also Figure S2.

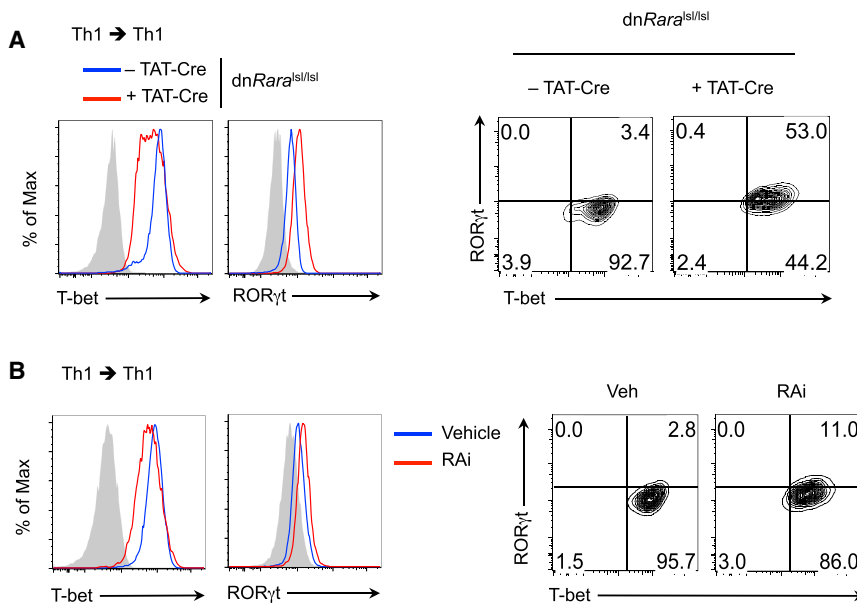


Figure 4. Loss of RA Signaling in Fully Committed Th1 Cells Leads to Th1 Plasticity and Divergence Toward the Th17 Lineage

(A) Naive CD4⁺ T cells from dnRara^{ts/1s1} mice were differentiated under Th1 conditions. Th1 cells were transduced with TAT-Cre on days 5 and 7 and re-polarized under Th1 conditions for a further 5 days. Intracellular expression of T-bet and RORγt.

(B) Naive CD4⁺ T cells from Ifng^{eYFP} mice were differentiated under Th1 conditions. IFN-γ (eYFP⁺) cells were sorted on day 7 and restimulated under Th1 conditions for 5 days in the presence of Veh or RAI. Intracellular expression of T-bet and RORγt. Data representative of two independent experiments.

See also Figure S4.

Th1-cell polarizing conditions. eYFP⁺ (IFN-γ⁺) cells were FACS-sorted on day 7 of culture and restimulated under Th1-cell conditions in the presence of the RAR inhibitor LE540 (RAi) or vehicle control (Veh). Inhibition of RA signaling in fully committed Th1 cells propagated for a further 5 days under Th1 conditions resulted in downregulation of T-bet and the emergence of cells co-expressing RORγt (Figure 4B). Diminished T-bet expression was associated with modest reductions in IFN-γ expression (Figure S4B). Taken together, these data establish that loss of RA signaling in fully committed Th1 cells leads to transdifferentiation to progeny with features of the Th17 lineage and support a model where RA constrains late-stage plasticity of Th1 cells.

RA-RARα Regulates Enhancer Activity at Lineage Determining Th1-Cell Genes

To better understand the molecular mechanism by which RARα regulates Th-cell fate, we performed genome-wide analysis of RARα binding in WT Th1 cells by ChIP-Seq, combined with transcriptional profiling of dnRara-expressing Th1 cells in order to identify functional targets of RARα. Selected loci were validated by ChIP-qPCR. RARα binding was identified at 1,766 sites in 1,567 genes. RARα binding was detected at 10.3% (76 of 740 genes) of genes downregulated in the absence of RA signaling (Table S2) (hereafter referred to as positively regulated) and 4.8% (56 of 1,169) of the upregulated genes (Table S3). In keeping with its classical role as a positive regulator of transcriptional activation there was significant enrichment of RARα binding at genes positively regulated by RA (Fisher's exact test, $p < 0.0001$). However, the presence of RARα at a subset of the negatively regulated genes indicates that RA-RARα also plays a role in transcriptional repression within Th1 cells.

RA-RARα-dependent loci included Th1-cell lineage-defining genes (*Tbx21* and *Stat4-Stat1*). In addition to targeting the *Tbx21* promoter (Figures 5A and 5B), modest RARα binding was observed at the conserved T-bet enhancer element, 12kb upstream of the transcriptional start site (TSS) (Yang et al., 2007). This was confirmed by ChIP-qPCR (Figure 5B). Intergenic

containing the histone acetyl-transferases p300 and CBP (Kamei et al., 1996). p300 is highly enriched at enhancer regions where it acetylates H3K27, a marker of active enhancers (Rada-Iglesias et al., 2011), suggesting a possible role for RA-RARα in regulating enhancer activity. To test this, we mapped genome-wide binding of p300, H3K4me1, H3K4me3, and H3K27ac histone modifications in dnRara and WT Th1-cells, validating selected regions by ChIP q-PCR. Active enhancers were operationally defined as regions with increased intensity of H3K4me1, p300, and H3K27ac with low or absent H3K4me3 (Rada-Iglesias et al., 2011).

RARα binding at the *Tbx21*, *Stat4*, and *Ifng* loci co-localized with p300 binding at enhancer regions (Figures 5A and S5A). dnRARA lacks the activation function 2 (AF2) domain which is required for RA-dependent recruitment of coactivators. Consistent with this, dnRara expressing T cells exhibited a significant reduction in p300 occupancy and H3K27ac deposition at the *Tbx21* enhancer, supporting the direct regulation of enhancer activity by RA-RARα (Figures 5A and 5C). p300 binding at the *Ifng* and putative *Stat4* intergenic enhancers was also dependent on RA-RARα (Figures S5A and S5C). Loss of p300 binding at the *Stat4-Stat1* intergenic enhancer in dnRara Th1 cells correlated with reduced *Stat4* transcripts, whereas *Stat1* expression was actually increased, suggesting that this enhancer element regulated *Stat4* transcription. A recent study identified a role for STAT4 in the regulation of Th1 enhancers (Vahedi et al., 2012). Given that STAT4 expression was reduced in dnRara Th1 cells, it was possible that the loss of p300 was in part due to reduced expression of STAT4. To address this issue, we assessed the binding of STAT4 in WT Th1 cells and compared p300 occupancy in WT and *Stat4*^{-/-} Th1 cells using publicly available ChIP-seq data (Table S1) (Vahedi et al., 2012; Wei et al., 2010). Although STAT4 binding was observed at the *Tbx21* enhancer, loss of STAT4 was not associated with obvious differences in p300 binding (Figure S5D), arguing for a direct contribution of RARα to p300 recruitment and enhancer activity. Collectively, these data show that RA regulates expression of key Th1-cell lineage genes through remodeling of enhancer regions.

RA-RAR α Represses Th17-Cell Fate in Th1 Cells through Direct Regulation of Th17-Cell Genes

The earlier finding that Th1 cells acquired features of Th17 cells in the absence of RA signaling led us to evaluate direct regulation of Th17-cell-instructing genes by RA-RAR α . We first investigated effects of RA on the Th17-cell pioneer factors BATF and IRF4. As previously reported (Basu et al., 2013), these genes were expressed in WT Th1 cells. Strikingly, kinetic analysis of *Batf* and *Irf4* expression in naive cells stimulated under Th1-cell conditions revealed dramatic upregulation of IRF4 (40- to 60-fold) during the initial phase of Th1-cell polarization with comparable expression between dnRara and WT cells (Figure 5D). Loss of RA signaling resulted in derepression of BATF-IRF4 target genes, *Rorc*, *Il23r*, *Il22*, *Il21*, and *Il12rb1* (Figure 5E). This suggested that “balancing” factors must be induced in an RA-dependent manner to restrict the actions of BATF-IRF4 complexes at Th17-cell genes. IRF8, an alternative binding partner for BATF, previously shown to suppress Th17 differentiation (Ouyang et al., 2011), was one of the RAR α target genes most suppressed in dnRara Th1 cells. In WT Th1 cells, induction of *Irf8* expression paralleled *Irf4* expression. However, in dnRara cells *Irf8* expression was not sustained past 24 hr (Figure 5D). RAR α bound at a putative upstream enhancer (Figures 5F and 5G) and in the absence of RA signaling, reduced p300 and H3K27ac were observed at this locus (Figure 5H and 5I). Together, these data show that RA directly regulates expression of IRF8 in Th1 differentiating cells and suggests a potential mechanism by which BATF-IRF4 activity is constrained within early Th1 cells.

Transcriptional activation of BATF-IRF4 target genes is dependent on STAT3 and ROR γ t (Ciofani et al., 2012). Various genes for cytokines and cytokine receptors associated with STAT3 activation (*Il21*, *Il1r1*, *Il6ra*, and *Il23r*) were derepressed in dnRara Th1 cells (Figure 5E). RAR α targeted the promoter and an upstream enhancer in the *Il6ra* locus (Figure 5G) with increased H3K27ac observed at the enhancer element in dnRara Th1 cells (Figure 5J). Consistent with this, dnRara Th1 cells failed to down-regulate mRNA and cell-surface IL6-R α expression during Th1 polarization (Figures S5E and S5F). These findings suggest that RA regulates Th1-cell plasticity in part by inhibiting responsiveness to IL-6.

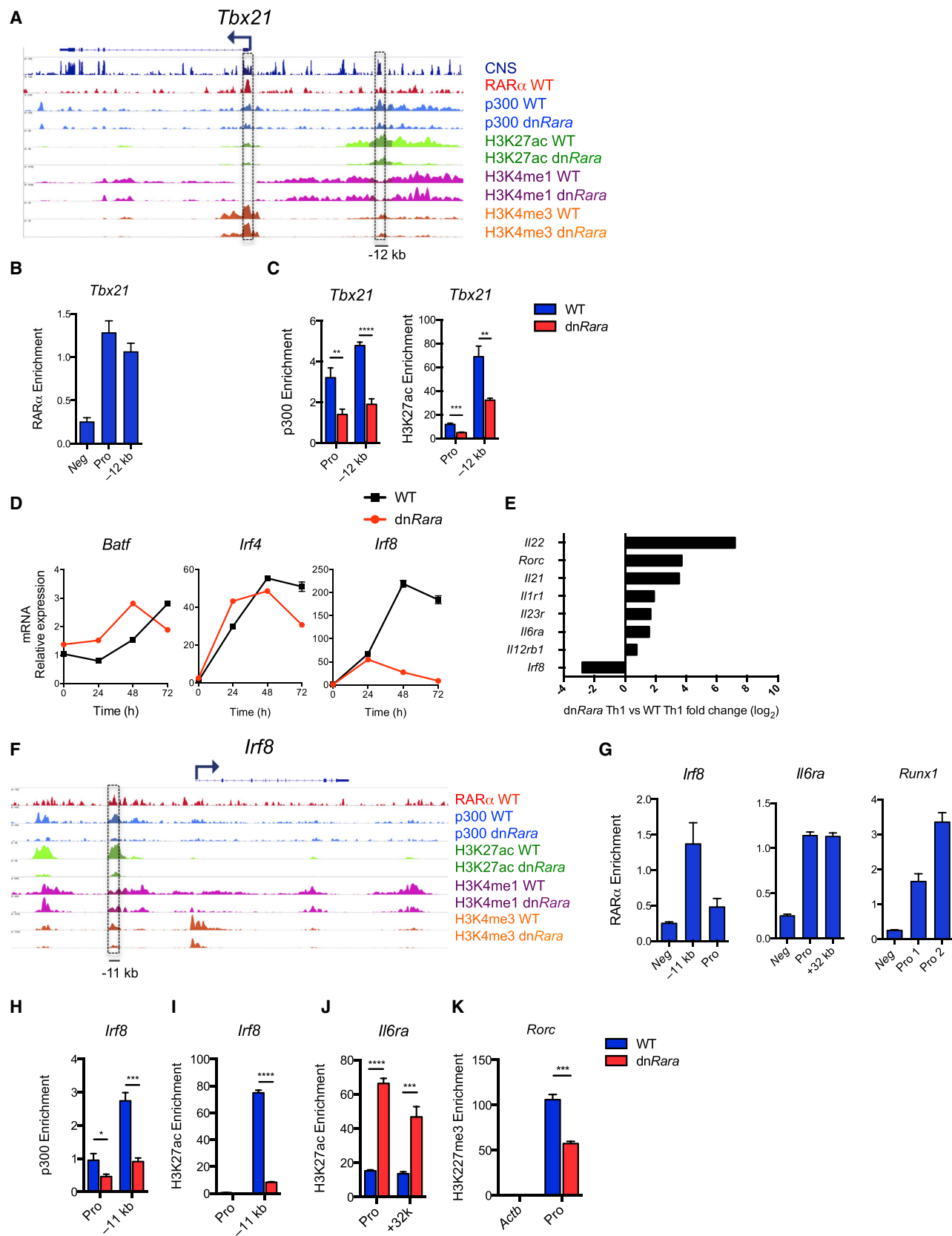
ROR γ t was not a direct target of RAR α . However, disruption of RA signaling resulted in increased expression of *Runx1*, a TF associated with transactivation of *Rorc* (Figure S5E) (Zhang et al., 2008). ChIP analysis confirmed direct regulation of short and long *Runx1* isoform promoters by RA-RAR α (Figure 5G). In Th1 cells, the *Rorc* locus is epigenetically silenced by T-bet (Mukasa et al., 2010). However, in dnRara cells, the repressive H3K27me3 mark was reduced at ROR γ t isoform-specific exon (Figure 5K), consistent with loss of T-bet. These findings suggest that increased ROR γ t expression in the absence of RAR α signaling is in part due to increased accessibility of the *Rorc* locus, with unrestrained activation by Runx1. Collectively these data indicate that RA-RAR α antagonizes the activity of the core Th17-cell instructing TFs (IRF4, BATF, STAT3, and ROR γ t), both directly and indirectly, to suppress the Th17-cell gene program. Notably, Th2-cell-associated genes were not identified as targets of RAR α (Tables S2 and S3) suggesting that direct repression of alternative cell fates by RA-RAR α is specific to the Th17-cell program.

Th1-like Th17 Cells Emerge during Infection with *L. monocytogenes* in the Absence of RA Signaling

To assess the significance of these findings for immune responses in vivo, we intravenously infected WT and dnRara mice with an attenuated strain of *L. monocytogenes* (Δ ActA), Lm-2W, which allows tracking of CD4 $^{+}$ T cells specific for listeriolysin O peptide LLO_{190–201} (LLOp). At the peak of the response, CD4 $^{+}$ T cells were isolated from the spleen and LLOp antigen-specific T cells were assayed for expression of cytokines and the TFs, T-bet, and ROR γ t. dnRara mice mounted an effector-T-cell response of similar magnitude to WT mice with comparable frequencies and total numbers of CD44 hi LLOp:I-A b -specific CD4 $^{+}$ T cells (Figures 6A and 6B). In WT mice, Lm-2W induced a Th1-cell restricted response, as evidenced by high T-bet expression within the LLOp-specific T-cell fraction (Figure 6C). LLOp:I-A b CD4 $^{+}$ T cells from dnRara mice expressed lower amounts of T-bet and a substantial proportion expressed ROR γ t, with co-expression of these TFs observed in a subset of cells (Figure 6C). At day 7 post-infection, a significant proportion of CD4 $^{+}$ T cells isolated from the spleen of dnRara mice were IL-17 $^{+}$ or dual IL-17A $^{+}$ IFN- γ $^{+}$ with a trend toward reduced frequency of IFN- γ $^{+}$ cells (Figure 6D). Measurement of cytokine protein concentrations from splenocytes restimulated with LLOp confirmed reduced amounts of IFN- γ and concomitant increase in IL-17A (Figure S6A). We did not detect IL-4 production by intracellular staining or protein secretion (Figure S6A and S6B). Consistent with our in vitro data showing downregulation of IL6-R α on WT Th1 cells, cell-surface IL6-R α was not detectable on WT LLOp:I-A b CD4 $^{+}$ T cells. However, dnRara LLOp:I-A b CD4 $^{+}$ T cells retained expression of IL6-R α (Figure S6C), supporting a potential role for IL-6 signaling in the regulation of Th1-cell plasticity. These findings establish that RA-RAR α signaling in T cells constrains the emergence of Th17 cells in a Th1-cell-instructing micro-environment in vivo.

RA Regulates the Th1-Th17-Cell Axis in the Gut and Prevents the Development of Intestinal Inflammation

RA is constitutively synthesized by a subset of DCs in the gut. To address the physiological importance of RA signaling in the regulation of pathogenic intestinal CD4 $^{+}$ T cells, we interbred dnRara mice with OTII mice that transgenically express an ovalbumin (OVA)-specific TCR and transferred naive CD4 $^{+}$ T cells from OTII(dnRara) or WT OTII mice into *Rag1* $^{-/-}$ hosts. Recipients were maintained on an OVA-containing diet for 7 days to induce differentiation within the transferred cells and migration to the intestinal tissue. Consistent with the infection experiments, feeding OTII(dnRara)-recipient mice OVA resulted in a shift in the Th1-Th17-cell balance with a deficiency in IFN- γ -producing cells and increased frequency of IL-17 $^{+}$ and dual IFN- γ $^{+}$ IL-17 $^{+}$ cells in the mesenteric lymph node (MLN), lamina propria lymphocytes (LPL), and spleen (Sp), 7 days after transfer (Figures 7B and 7C). To address the functional significance of the dysregulated cytokine response in dnRara T cells, we orally challenged mice with OVA and evaluated them for development of intestinal inflammation and diarrhea (Figure 7A). Recipients of OTII(dnRara) cells developed accelerated wasting disease relative to mice that received WT OTII cells (Figure 7D). Whereas all of the recipients of OTII(dnRara) cells developed severe diarrhea



(legend on next page)

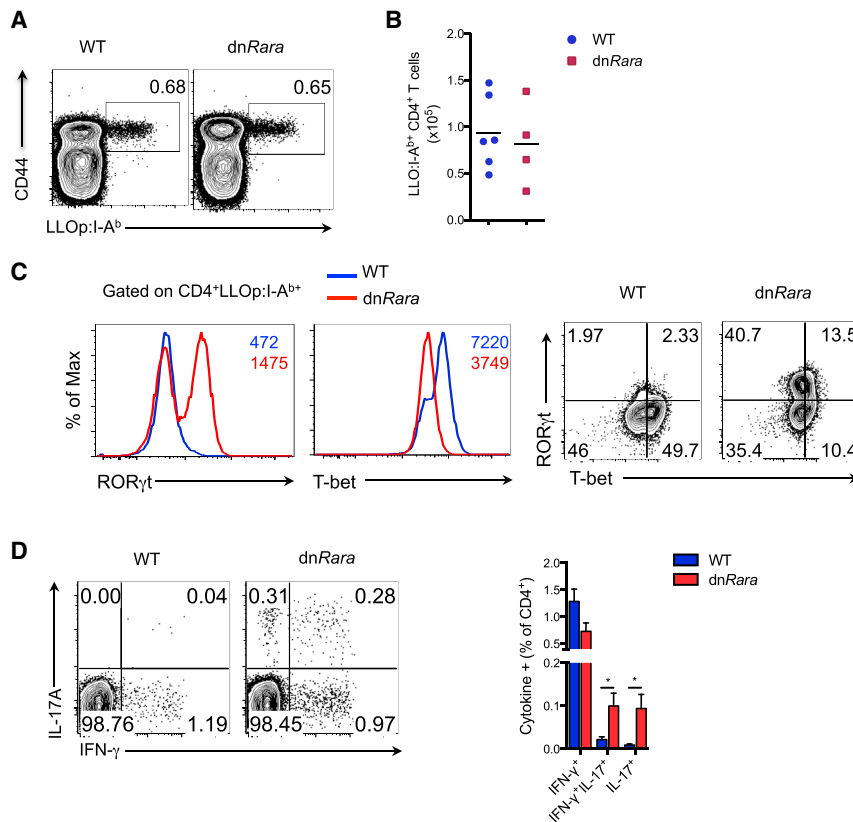


Figure 6. RA Signaling Required to Prevent the Generation of Th17 Cells during Infection with *L. monocytogenes*

(A) Frequency of LLOp:I-A^b CD4⁺ T cells isolated from spleen of dnRara and WT mice 7 days after infection with an attenuated strain of *L. monocytogenes* (Lm-2W). Gated on CD4⁺ T cells.

(B) Absolute numbers of LLOp:I-A^b CD4⁺ T cells as in (A).

(C) Intracellular T-bet and RORγt expression gated on LLOp:I-A^b CD4⁺ T cells.

(D) Intracellular staining for IFN-γ and IL-17A following stimulation of splenocytes with LLOp for 6 hr, 7 days after infection with Lm-2W. Gated on CD4⁺ T cells. Right panel shows statistical data pooled from three independent experiments (3–6 mice per group).

Representative data of at least three (A and B), or two independent experiments (C). Mean ± SEM. See also Figure S6.

DISCUSSION

Dysregulated Th-cell responses underlie the pathogenesis of autoimmune and allergic disease. In contrast to T regulatory (Treg) cells and Th17 cells, the Th1-cell lineage is thought to be relatively stable. However, the factors that control maintenance of the Th1-cell lineage were not

previously known. This study identifies RA-RARα as a central regulatory node in the transcriptional network governing Th1-cell stability. We found that RA-RARα directly sustained the expression of lineage determining Th1-cell-associated genes during naive T-cell differentiation while also repressing signature Th17-cell-associated genes. Ablation of RA signaling in Th1-committed cells resulted in enhanced Th1-cell plasticity with deviation towards a Th17-cell phenotype. Using ChIP-seq to identify regulatory elements, we found that RARα bound at enhancers and recruitment of p300 to these regions was dependent on RA signaling. In vivo, both Th17 and Th1-Th17 cells emerged during infection with *L. monocytogenes* and in a model

by day 12 (Figure 7E), recipients of WT cells remained diarrhea free. Cytokine production was also assessed after the first gavage and confirmed an increased frequency of IL-17⁺ cells with concomitant reduction in IFN-γ⁺ cells. Notably, enhanced IL-17 responses were not a consequence of impaired Foxp3⁺ conversion (Figure 7F). Homing of transferred cells to the gut was not affected in this model with similar frequencies of CD4⁺ T cells detected in the gut tissues (Figure S7A). We conclude that loss of RA signaling leads to deviation from Th1 to Th17 phenotype both in the periphery and the gut where these Th17 cells are associated with significant intestinal inflammation.

Figure 5. RA-RARα Regulates Enhancer Activity at Th1 Lineage Associated Loci and Represses Th17 Genes

Naive CD4⁺ T cells from WT and dnRara mice were cultured for 6 days under Th1 conditions prior to chromatin precipitation and transcriptional profiling.

(A) ChIP-seq binding tracks at *Tbx21* locus for RARα in WT Th1 cells and p300 binding, H3K27ac, H3K4me1, and H3K4me3 modifications in WT and dnRara Th1 cells.

(B) Validation of the RARα-binding regions in WT Th1 cells by ChIP-qPCR. Untr6 region serves as a negative control. Binding events per 1,000 cells displayed as "Enrichment."

(C) The effects of dnRara expression on p300 and H3K27ac abundance at the *Tbx21* locus were validated by ChIP-qPCR.

(D) Quantitative real-time PCR analysis of *Batf*, *Irf4*, and *Irf8* mRNA in naive CD4⁺ T cells from dnRara or WT cells differentiated under Th1-cell conditions for 0, 24, 48, 72 hr. Mean ± SEM, replicate wells.

(E) Log2 values of fold changes in gene expression as measured by microarray analyses. Average fold change depicted.

(F) ChIP-seq binding tracks at *Irf8* locus for cells as in (A).

(G) Validation of RARα ChIP-seq regions by ChIP-qPCR.

(H–J) ChIP analysis of p300 and H3K27ac at selected loci.

(K) ChIP analysis of H3K27me3 at the RORc locus. *Actb* locus serves as a negative control.

Data from three independent experiments (E) or representative of two independent experiments (B–D, G–K); Mean ± SD unless noted otherwise. Abbreviation: pro, promoter.

See also Figure S5.

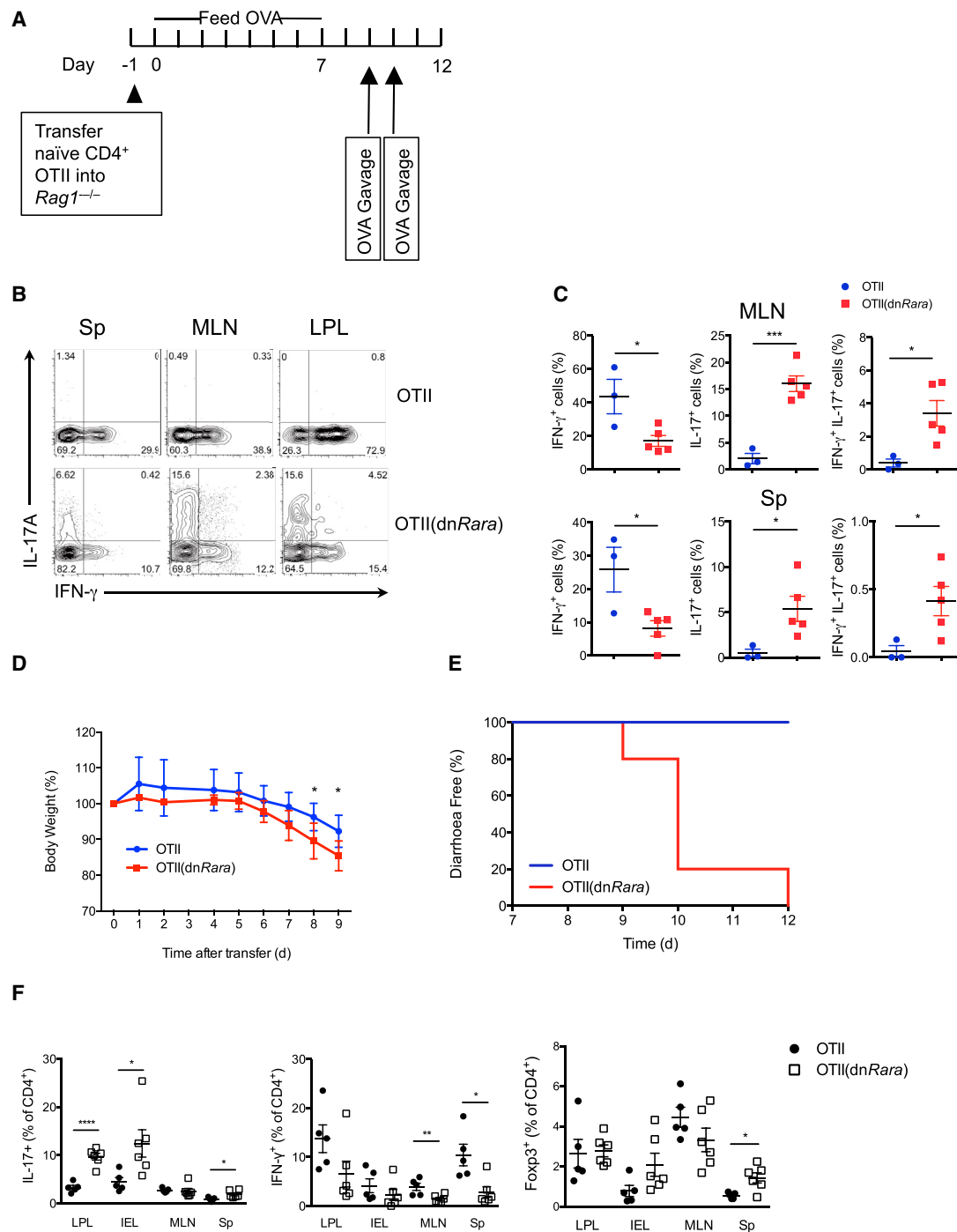


Figure 7. Loss of RA Signaling Causes Dysregulated Th1 and Th17 Response and Increased Pathogenicity in a Model of Gut Inflammation

(A) Schematic illustration of the adoptive transfer experiment.

(B) Intracellular expression of IL-17A and IFN-γ among CD4⁺ cells from the spleen (Sp), mesenteric lymph nodes (MLN), and lymphocytes from the lamina propria (LPL) of mice as in (A) 7 days after transfer.

(C) Statistical data for frequency of IFN-γ⁺, IL-17⁺, and IFN-γ⁺IL-17⁺ cells as in (B) in MLN and Sp.

(D) Percentile change of original body weight in *Rag1*^{-/-} recipients treated as in (A) (n = 5–7 per group). Mean ± SD.

(E) Frequency of diarrhea-free mice among *Rag1*^{-/-} recipients as in (A) (OTII recipients n = 3, OTII(dnRara) recipients n = 5).

(F) Frequencies of IL-17, IFN-γ, and Foxp3 in CD4⁺ cells isolated from Sp, MLN, LPL, and IELs of mice as in (A), 9 days after transfer (n = 5 or 6 per group).

Data from one experiment (B and C), pooled from two independent experiments (D and F), or representative of two independent experiments (E). Mean ± SEM unless otherwise noted.

of oral tolerance. In the latter, their presence was associated with significant pathology.

Enhancers play a key role in directing cell fate through the regulation of lineage specifying genes. Enhancer profiling in WT and *dnRara* T cells revealed RA-dependent activation of enhancers at genes critical for Th1 identity (*Tbx21*, *Stat4*, *Ifng*, and *Irf8*). RA-dependent changes in p300 and H3K27ac were reflected at the transcriptional level suggesting that, in addition to its classical role as a transcriptional regulator, RA regulates gene expression in an enhancer-dependent manner. Although the ability of RA-RAR α to target p300-CBP complexes to nucleosomes is well established, regulation of enhancers by RA has not been widely studied. We propose that unliganded RAR α at enhancer elements acts as a gatekeeper, enabling initiation of enhancer activation once T cells sense RA in the microenvironment. A similar role has been demonstrated for STAT proteins (Vahedi et al., 2012), suggesting that environmental cues act as checkpoints for initiation of enhancer activation and T-cell fate. Although H3K4me1 modifications are present at early time points during T-cell differentiation, conversion to “active” status requires acquisition of H3K27ac, which is often not evident until later stages of differentiation (Hawkins et al., 2013). Consistent with a temporal role for enhancers in maintenance of gene expression, RA signaling was not required for initiation of transcription of target genes but rather acted to maintain their expression. These data highlight the importance of enhancers in maintenance of cell identity and plasticity. It is possible that RA-RAR α regulation of enhancers represent the major mechanism by which RA regulates cell fate. A recent study identified enrichment of RAR α at enhancers in embryonic stem cells (Chen et al., 2012). Given that the RA-RAR α axis is a highly conserved signaling pathway, which plays a critical role in regulating cell-fate specification during embryogenesis and cell differentiation, it will be important to evaluate a broader role for RA-RAR α in regulation of enhancer functionality, both in alternative Th-cell subsets and outside of the immune system.

In addition to sustaining expression of Th1-cell-associated genes, we found that RA actively silences genes implicated in Th17-cell differentiation. Among genes known to regulate the Th17-cell program, *Runx1* and *Il6ra* were directly repressed by RA-RAR α . In addition, BATF-IRF4 target genes were derepressed in the absence of RA signaling. In Th17 cells, BATF-IRF4 complexes act co-operatively as pioneer factors at key Th17 genes (Ciofani et al., 2012), modulating chromatin accessibility to facilitate binding of STAT3 and ROR γ t. On the basis of their expression in alternative Th-cell subsets, it has been suggested that BATF-IRF4 complexes play a universal role in establishing binding of lineage-specific TFs (Ciofani et al., 2012). However, BATF deficiency does not impact on Th1-cell differentiation (Schraml et al., 2009). An alternative model is that upregulation of BATF and IRF4 confers plasticity in early Th1 cells, poising chromatin specifically at Th17-cell-associated genes. IRF8, an alternative binding partner for BATF, negatively regulates Th17-cell differentiation (Ouyang et al., 2011). Our results identified IRF8 as a member of the Th1-cell transcriptional network whose expression was critically dependent on RA signaling. Induction of IRF8 would be expected to limit plasticity of Th1 cells by repressing Th17 differentiation, potentially by competing for binding to BATF. In support of a role for IRF8 in

regulation of Th1-Th17 axis, patients with mutations in IRF8 have impaired Th1 responses (Hambleton et al., 2011) and single nucleotide polymorphisms (SNPs) in *Irf8* are associated with several autoimmune diseases in which IFN- γ ⁺ Th17 cells play a pathogenic role (Franke et al., 2010; Cunniff et al., 2011). It will be of interest to identify transcriptional targets of BATF, IRF4, and IRF8 in Th1 cells.

RA signaling was critical to maintain appropriate Th1-cell responses and suppress the development of IL-17⁺ and IFN- γ ⁺IL-17⁺ cells. Hybrid Th1-Th17 cells are implicated in the pathogenesis of several autoimmune diseases. Their development has been attributed to the plasticity of Th17 cells. Our findings suggest that these cells might alternatively reflect Th1 plasticity and suggest a novel developmental pathway for Th17 cells. Th1 derived “Th17” cells expressed high levels of the receptor for IL-23, a critical determinant of Th17 pathogenicity (Basu et al., 2013), and were associated with significant gut inflammation and pathology in a model of oral tolerance. Further experiments are required to test the prediction that pathogenic Th17 and IFN- γ ⁺IL-17⁺ cells which arise in autoimmunity emerge from Th1 cells when RA is deficient or its signaling perturbed.

A range of inflammatory stimuli can induce RA synthesis and signaling during the course of an immune response. Our results suggest that in a Th1-cell instructing microenvironment the dominant action of RA is to repress Th17-cell fate and promote Th1-cell responses. We did not observe enhanced Th17-cell responses during primary Th17-cell differentiation, suggesting that the impact of RA on T-cell stability might vary both temporally and among tissues. Previously we have shown in a model of skin allograft rejection that impaired Th1 responses in *dnRara* mice were accompanied by increased Th2-cell cytokines (Pino-Lagos et al., 2011). We did not identify direct repression of Th2-cell-associated genes by RAR α . However, T-bet suppresses GATA3 (Zhu et al., 2012) and in the presence of a Th2 skewing microenvironment, such as the skin, impaired expression of T-bet in the absence of RA signaling renders cells susceptible to Th2 deviation. Thus, the effects of RA on T-cell fate are likely dependent on external and intrinsic factors that shape T-cell polarity.

In summary, we show that RA signaling plays a critical role in regulating stability and functional plasticity of Th1 cells. Regulation of enhancer activity at lineage determining genes by RA-RAR α provides mechanistic evidence for reciprocal regulation of Th1 and Th17-cell programs. In the absence of RA signaling, downmodulation of T-bet, STAT4, and IFN- γ , and loss of repression of Th17-cell genes, creates a permissive environment for transdifferentiation of Th1 cells to Th17 cells. This study identifies the RA-RAR α axis as a potential node for intervention in diseases in which dysregulation of the Th1-Th17-cell axis is observed.

EXPERIMENTAL PROCEDURES

Mice

C57Bl/6 *dnRara* mice have been described previously (Pino-Lagos et al., 2011). *Ifng*^{eYFP} (GREAT) mice were purchased from the Jackson Laboratory. Mice were bred and maintained at Charles River Laboratory, UK, in pathogen-free conditions. All animal experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986. C57Bl/6 OTII(*dnRara*), OTII, and *Rag1*^{-/-} mice were bred and maintained at the Rockefeller University specific pathogen-free animal facility.

Cell Isolation, Cell Culture, and Flow Cytometry

Sort purified, naive CD4⁺CD25[−]CD44^{lo}CD62L^{hi} T cells were cultured with T-cell depleted splenocytes (APCs) and anti-CD3 under polarization conditions for Th0, Th1, Th2, and Th17-cell-associated subsets. Details are provided in the [Supplemental Experimental Procedures](#). For analysis of cytokine production, cells were restimulated with 100 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin in the presence of monensin for 4–5 hr at 37°C. Cells were stained with LIVE/DEAD Dead Cell Stain (Invitrogen), followed by staining for cell-surface markers and then fixed and permeabilized (BD Biosciences) for staining of intracellular antigens. Flow cytometry was performed on a LSR Fortessa (BD Biosciences) and analyzed with Flowjo software (Tree Star).

TAT-Cre Transduction

Sort purified naive CD4⁺ T cells were differentiated under Th1 conditions. After 5 days, cells were treated with 50 µg/ml TAT-Cre peptide for 45 min at 37°C, then washed and expanded in IL-2-containing medium. After 48 hr cells were retreated with Tat-Cre followed by polarization under Th1 conditions.

Real-Time Quantitative PCR

Total RNA was extracted from cells with RNeasy Mini kit (QIAGEN) and cDNA was synthesized with Qscript RT kit (Quanta). Quantitative gene-expression analysis was performed using Taqman primer probe sets (Applied Biosystems), listed in [Table S4](#). Expression of target genes was normalized to β-actin.

Microarray Gene-Expression Profiling

For gene-expression analysis Affymetrix (for *Irfng*^{eYFP} dataset) or Agilent (for the *dnRara* Th1 dataset) microarray chips were used. Differentially expressed genes were detected using fold-change and t test analysis. See [Supplemental Experimental Procedures](#) for further information.

Chromatin Immunoprecipitation and ChIP-Seq

Immunoprecipitation and DNA sequencing was performed by Active Motif. The following antibodies were used: anti-H3K27me3 (Millipore 07-449), anti-p300 (Santa Cruz sc-551X), anti-H3K4me1 (Active Motif 39287), anti-H3K4me3 (Active Motif 39159), anti-H3K27ac (active Motif 39133), anti-RARα (Diagenode C15310155). Illumina sequencing libraries were prepared from the ChIP and Input DNAs. For ChIP q-PCR, enrichment calculated as binding events per 1,000 cells using Active Motif's normalization scheme. Detailed methods for ChIP-seq and binding site analyses are provided in the [Supplemental Information](#).

L. monocytogenes Infection

Mice were infected i.v. with 1×10^6 cfu *L. monocytogenes* and spleens were harvested 7 days later. Splenocytes were enriched for CD4⁺ T cells with a CD4⁺ T-cell negative selection microbead kit (Miltenyi Biotec) and stained with PE labeled, LLO:I-A^b dextramer (Immudex) and cell-surface antibodies. For analysis of intracellular cytokine production, splenocytes were restimulated with LLO peptide (PiProteomics) at 10 µg/ml for 6 hr.

Food-Antigen-Induced Diarrhea Model

Naive CD4⁺ T cells from OTII or OTII(*dnRara*) were intravenously transferred to Rag1^{−/−} mice. These mice were then maintained on a diet containing OVA for 7 days and challenged with oral OVA on days 9 and 10. Lymphocytes were isolated from the intestinal epithelium, lamina propria, MLN, and spleen at the indicated time points after the start of oral OVA exposure of the recipient mice. Detailed experimental procedures are described in the [Supplemental Experimental Procedures](#).

Statistical Analysis

Statistical significance was calculated by unpaired two-tailed Student's t test with Graphpad Prism software. p values < 0.05 were considered significant. p values are denoted in figures as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001.

ACCESSION NUMBERS

Chip-seq and microarray data are available under GEO accession number GSE60356.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, five tables, and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2015.02.003>.

AUTHOR CONTRIBUTIONS

C.C.B. designed the studies, performed most of the experiments, analyzed the data, and wrote the manuscript. D.E., M.L., and D.M. performed and analyzed gut-inflammation studies. A.S., I.O.-G., and R.a.-B. assisted in processing of samples for in vitro co-culture studies and qPCR. R.E. and C.O. assisted with processing of tissues for phenotyping studies. M.A. provided advice and performed microarrays. V.P. analyzed ChIP sequencing data. E.d.R. supervised ChIP-seq data analysis. G.M.L. provided advice and supervision. D.M. designed gut-inflammation studies and contributed to the writing of the manuscript. R.J.N. supervised the overall study and contributed to the writing of the manuscript.

ACKNOWLEDGMENTS

We are indebted to PJ Chana and H. Graves (BRC Flow Cytometry Core, Kings College) for cell sorting. We gratefully acknowledge the NIH Tetramer Core Facility for provision of I-A^b:LLO_{190–201} monomers and thank M. Jenkins (University of Minnesota) for providing us with Lm-2W. We thank K. Jones for critical reading of the manuscript and A. Hertweck for advice on experiments. This work was supported by a Wellcome Trust Research Training Fellowship to C.C.B. and a Wellcome Trust Principal Research Fellowship Award and NIH R01AT005382 award to R.J.N. Research was supported in part by the National Institute for Health Research (NIHR) Biomedical Research Centre, Guy's and St Thomas' National Health Service (NHS) Foundation Trust and King's College London and the Medical Research Council (MRC) Centre for Transplantation, King's College London, UK-MRC grant no. MR/J006742/1. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR, or the Department of Health.

Received: August 16, 2014

Revised: December 18, 2014

Accepted: January 16, 2015

Published: March 10, 2015

REFERENCES

- Annunziato, F., Cosmi, L., Santarlasci, V., Maggi, L., Liotta, F., Mazzinghi, B., Parente, E., Fili, L., Ferri, S., Frosali, F., et al. (2007). Phenotypic and functional features of human Th17 cells. *J. Exp. Med.* 204, 1849–1861.
- Aoyama, K., Saha, A., Tolar, J., Riddle, M.J., Veenstra, R.G., Taylor, P.A., Blomhoff, R., Panoskaltsis-Mortari, A., Klebanoff, C.A., Socié, G., et al. (2013). Inhibiting retinoic acid signaling ameliorates graft-versus-host disease by modifying T-cell differentiation and intestinal migration. *Blood* 122, 2125–2134.
- Basu, R., Hatton, R.D., and Weaver, C.T. (2013). The Th17 family: flexibility follows function. *Immunol. Rev.* 252, 89–103.
- Chanda, B., Ditadi, A., Iscove, N.N., and Keller, G. (2013). Retinoic acid signaling is essential for embryonic hematopoietic stem cell development. *Cell* 155, 215–227.
- Chen, C.Y., Morris, Q., and Mitchell, J.A. (2012). Enhancer identification in mouse embryonic stem cells using integrative modeling of chromatin and genomic features. *BMC Genomics* 13, 152–170.
- Ciofani, M., Madar, A., Galan, C., Sellars, M., Mace, K., Pauli, F., Agarwal, A., Huang, W., Parkurst, C.N., Muratet, M., et al. (2012). A validated regulatory network for Th17 cell specification. *Cell* 151, 289–303.
- Cunningham-Graham, D.S., Morris, D.L., Bhangale, T.R., Criswell, L.A., Syvänen, A.-C., Rönnblom, L., Behrens, T.W., Graham, R.R., and Vyse, T.J. (2011). Association of NCF2, IKZF1, IRF8, IFIH1, and TYK2 with systemic lupus erythematosus. *PLoS Genet.* 7, e1002341.

- Franke, A., McGovern, D.P.B., Barrett, J.C., Wang, K., Radford-Smith, G.L., Ahmad, T., Lees, C.W., Balschun, T., Lee, J., Roberts, R., et al. (2010). Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nat. Genet.* **42**, 1118–1125.
- Hall, J.A., Cannons, J.L., Grainger, J.R., Dos Santos, L.M., Hand, T.W., Naik, S., Wohlfert, E.A., Chou, D.B., Oldenhove, G., Robinson, M., et al. (2011). Essential role for retinoic acid in the promotion of CD4(+) T cell effector responses via retinoic acid receptor alpha. *Immunity* **34**, 435–447.
- Hambleton, S., Salem, S., Bustamante, J., Bigley, V., Boisson-Dupuis, S., Azevedo, J., Fortin, A., Haniffa, M., Ceron-Gutierrez, L., Bacon, C.M., et al. (2011). IRF8 mutations and human dendritic-cell immunodeficiency. *N. Engl. J. Med.* **365**, 127–138.
- Hawkins, R.D., Larjo, A., Tripathi, S.K., Wagner, U., Luu, Y., Lönnberg, T., Raghav, S.K., Lee, L.K., Lund, R., Ren, B., et al. (2013). Global chromatin state analysis reveals lineage-specific enhancers during the initiation of human T helper 1 and T helper 2 cell polarization. *Immunity* **38**, 1271–1284.
- Irvin, C., Zafar, I., Good, J., Rollins, D., Christianson, C., Gorska, M.M., Martin, R.J., and Alam, R. (2014). Increased frequency of dual-positive TH2/TH17 cells in bronchoalveolar lavage fluid characterizes a population of patients with severe asthma. *J. Allergy Clin. Immunol.* **134**, 1175–1186.e7.
- Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S.C., Heyman, R.A., Rose, D.W., Glass, C.K., and Rosenfeld, M.G. (1996). A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* **85**, 403–414.
- Kashyap, V., Laursen, K.B., Brenet, F., Viale, A.J., Scandura, J.M., and Gudas, L.J. (2013). RAR γ is essential for retinoic acid induced chromatin remodeling and transcriptional activation in embryonic stem cells. *J. Cell Sci.* **126**, 999–1008.
- Kebir, H., Ifergan, I., Alvarez, J.I., Bernard, M., Poirier, J., Arbour, N., Duquette, P., and Prat, A. (2009). Preferential recruitment of interferon-gamma-expressing TH17 cells in multiple sclerosis. *Ann. Neurol.* **66**, 390–402.
- Klebanoff, C.A., Spencer, S.P., Torabi-Parizi, P., Grainger, J.R., Roychoudhuri, R., Ji, Y., Sukumar, M., Muranski, P., Scott, C.D., Hall, J.A., et al. (2013). Retinoic acid controls the homeostasis of pre-cDC-derived splenic and intestinal dendritic cells. *J. Exp. Med.* **210**, 1961–1976.
- Komatsu, N., Okamoto, K., Sawa, S., Nakashima, T., Oh-hora, M., Kodama, T., Tanaka, S., Bluestone, J.A., and Takayanagi, H. (2014). Pathogenic conversion of Foxp3+ T cells into TH17 cells in autoimmune arthritis. *Nat. Med.* **20**, 62–68.
- Mucida, D., Park, Y., Kim, G., Turovskaya, O., Scott, I., Kronenberg, M., and Cheroutre, H. (2007). Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science* **317**, 256–260.
- Mukasa, R., Balasubramani, A., Lee, Y.K., Whitley, S.K., Weaver, B.T., Shibata, Y., Crawford, G.E., Hatton, R.D., and Weaver, C.T. (2010). Epigenetic instability of cytokine and transcription factor gene loci underlies plasticity of the T helper 17 cell lineage. *Immunity* **32**, 616–627.
- Nistala, K., Adams, S., Cambrook, H., Ursu, S., Olivito, B., de Jager, W., Evans, J.G., Cimaz, R., Bajaj-Elliott, M., and Wedderburn, L.R. (2010). Th17 plasticity in human autoimmune arthritis is driven by the inflammatory environment. *Proc. Natl. Acad. Sci. USA* **107**, 14751–14756.
- Ouyang, X., Zhang, R., Yang, J., Li, Q., Qin, L., Zhu, C., Liu, J., Ning, H., Shin, M.S., Gupta, M., et al. (2011). Transcription factor IRF8 directs a silencing programme for TH17 cell differentiation. *Nat. Commun.* **2**, 314.
- Pino-Lagos, K., Guo, Y., Brown, C., Alexander, M.P., Elgueta, R., Bennett, K.A., De Vries, V., Nowak, E., Blomhoff, R., Sockanathan, S., et al. (2011). A retinoic acid-dependent checkpoint in the development of CD4+ T cell-mediated immunity. *J. Exp. Med.* **208**, 1767–1775.
- Rada-Iglesias, A., Bajpai, R., Swigut, T., Brugmann, S.A., Flynn, R.A., and Wysocka, J. (2011). A unique chromatin signature uncovers early developmental enhancers in humans. *Nature* **470**, 279–283.
- Reis, B.S., Rogoz, A., Costa-Pinto, F.A., Taniuchi, I., and Mucida, D. (2013). Mutual expression of the transcription factors Runx3 and ThPOK regulates intestinal CD4+ T cell immunity. *Nat. Immunol.* **14**, 271–280.
- Schraml, B.U., Hildner, K., Ise, W., Lee, W.-L., Smith, W.A.E., Solomon, B., Sahota, G., Sim, J., Mukasa, R., Cemerski, S., et al. (2009). The AP-1 transcription factor Batf controls T(H)17 differentiation. *Nature* **460**, 405–409.
- Schulz, E.G., Mariani, L., Radbruch, A., and Höfer, T. (2009). Sequential polarization and imprinting of type 1 T helper lymphocytes by interferon-gamma and interleukin-12. *Immunity* **30**, 673–683.
- Spencer, S.P., Wilhelm, C., Yang, Q., Hall, J.A., Bouladoux, N., Boyd, A., Nutman, T.B., Urban, J.F., Jr., Wang, J., Ramalingam, T.R., et al. (2014). Adaptation of innate lymphoid cells to a micronutrient deficiency promotes type 2 barrier immunity. *Science* **343**, 432–437.
- Takahashi, H., Kanno, T., Nakayama, S., Hirahara, K., Sciumè, G., Muljo, S.A., Kuchen, S., Casellas, R., Wei, L., Kanno, Y., and O'Shea, J.J. (2012). TGF- β and retinoic acid induce the microRNA miR-10a, which targets Bcl-6 and constrains the plasticity of helper T cells. *Nat. Immunol.* **13**, 587–595.
- Urvalek, A.M., and Gudas, L.J. (2014). Retinoic acid and histone deacetylases regulate epigenetic changes in embryonic stem cells. *J. Biol. Chem.* **289**, 19519–19530.
- Vahedi, G., Takahashi, H., Nakayama, S., Sun, H.-W., Sartorelli, V., Kanno, Y., and O'Shea, J.J. (2012). STATs shape the active enhancer landscape of T cell populations. *Cell* **151**, 981–993.
- Wadia, J.S., Stan, R.V., and Dowdy, S.F. (2004). Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat. Med.* **10**, 310–315.
- Wei, L., Vahedi, G., Sun, H.-W., Watford, W.T., Takatori, H., Ramos, H.L., Takahashi, H., Liang, J., Gutierrez-Cruz, G., Zang, C., et al. (2010). Discrete roles of STAT4 and STAT6 transcription factors in tuning epigenetic modifications and transcription during T helper cell differentiation. *Immunity* **32**, 840–851.
- Xiao, S., Jin, H., Korn, T., Liu, S.M., Oukka, M., Lim, B., and Kuchroo, V.K. (2008). Retinoic acid increases Foxp3+ regulatory T cells and inhibits development of Th17 cells by enhancing TGF-beta-driven Smad3 signaling and inhibiting IL-6 and IL-23 receptor expression. *J. Immunol.* **181**, 2277–2284.
- Yang, Y., Ochando, J.C., Bromberg, J.S., and Ding, Y. (2007). Identification of a distant T-bet enhancer responsive to IL-12/Stat4 and IFN γ /Stat1 signals. *Blood* **110**, 2494–2500.
- Zhang, F., Meng, G., and Strober, W. (2008). Interactions among the transcription factors Runx1, ROR γ and Foxp3 regulate the differentiation of interleukin 17-producing T cells. *Nat. Immunol.* **9**, 1297–1306.
- Zhou, L., Ivanov, I.I., Spolski, R., Min, R., Shenderov, K., Egawa, T., Levy, D.E., Leonard, W.J., and Littman, D.R. (2007). IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat. Immunol.* **8**, 967–974.
- Zhu, J., Jankovic, D., Oler, A.J., Wei, G., Sharma, S., Hu, G., Guo, L., Yagi, R., Yamane, H., Punkosdy, G., et al. (2012). The transcription factor T-bet is induced by multiple pathways and prevents an endogenous Th2 cell program during Th1 cell responses. *Immunity* **37**, 660–673.